

In vivo and in vitro genetic evidence of involvement of neuregulin 1 in immune system dysregulation

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Abstract Neuregulin 1 (NRG1) has been implicated in several disorders including breast cancer, multiple sclerosis, and schizophrenia. Also, recent evidence suggests that NRG1 may play a role in regulation of inflammation and immune system response. We therefore hypothesized that a schizophrenia-associated missense mutation (valine to

leucine) we identified within the transmembrane region of NRG1 would also be linked to immune dysregulation. We used plasma samples from families carrying the mutation to measure levels of antibodies to 41 autoimmune markers and six cytokines (IL-1b, IL-6, IL-10, IL-8, IL-12p70, and TNF- α) and used these levels as quantitative traits to evaluate association with the *NRG1* mutation, using FBAT. Next, we used Epstein–Barr virus-transformed B cells from heterozygous mutation carriers and wild-type individuals to evaluate protein and mRNA cytokine expression in vitro using quantitative PCR and ELISA assays. In vivo, increased levels of 25 autoimmune markers as well as elevated levels of cytokines were significantly associated with the *NRG1* mutation. In vitro, we observed a significant increase in protein secretion levels of IL-6, TNF- α , and IL-8 in mutation carriers compared with controls. At the mRNA level, we observed a significant increase in IL-6 expression, while IL-4 levels appeared to be down-regulated in heterozygous individuals compared with wild-type controls. This is the first report of association of a *NRG1* mutation with immune dysregulation. This study could contribute towards understanding the role of NRG1 in the pathogenesis of schizophrenia and other disorders in which inflammation plays an important role.

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Introduction

Neuregulin 1 (NRG1) mediates cell–cell interactions via activation of ErbB receptors, and is known to be involved in several biological processes, including cell proliferation, migration, and apoptosis, affecting multiple systems [1]. In

the brain, NRG1 plays roles in processes such as neuronal migration, gliogenesis, neurotransmitter expression, and myelination, which are thought to account for its involvement in brain disorders such as multiple sclerosis and schizophrenia. More recently, several lines of evidence have emerged, suggesting that NRG1 may also be implicated in regulation of the immune response [2, 4] possibly by functioning as an anti-inflammatory or antioxidant agent. Studies in rodents have shown that NRG1 has a neuroprotective role during conditions of ischemia or trauma [3]. It has been reported that human genetic variation in *NRG1* modulates the effects of stress on early atherosclerosis [4], a well-established inflammatory process. Also, NRG1 has been shown to influence cell adhesion of immune cells [5] and to attenuate the release of free radicals from microglial cells [2]. More recently, it was shown that in immune-deficient mice, the levels of a splice variant of NRG1 were decreased in medial prefrontal cortices, signifying a crosstalk between NRG1 and the immune system [6].

Immune system abnormalities have been observed in patients with psychiatric disorders such as schizophrenia [7, 8], as evidenced by increased expression of proinflammatory cytokines and high levels of autoantibodies. Incidence of suffering from an autoimmune disease is correlated with a 45% increase in risk for schizophrenia [9]. The mechanism by which the immune system may contribute to development of psychiatric disorders is not well understood.

We previously identified a novel *NRG1* mutation associated with schizophrenia in the founder population of the Central Valley of Costa Rica (CVCR). This mutation causes the change of a residue (valine to leucine, $G>T$) in the transmembrane domain of the NRG1 protein [10]. The transmembrane domain is thought to be important for cleavage of NRG1 via metalloproteases and secretases. Recently, it was shown that the $V>L$ mutation affects gamma-secretase cleavage of the intracellular domain of

NRG1 [11], which has been shown to act as a transcription modulator [12]. This suggests the relevance of the $V>L$ mutation in NRG1 protein function.

Given the involvement of NRG1 in immune system response and the apparent importance of the $V>L$ mutation in NRG1 cleavage, we hypothesized that this mutation could contribute to immune system dysregulation, thereby possibly playing a role in development of immune-related disorders, such as schizophrenia.

Materials and methods

Ethics statement

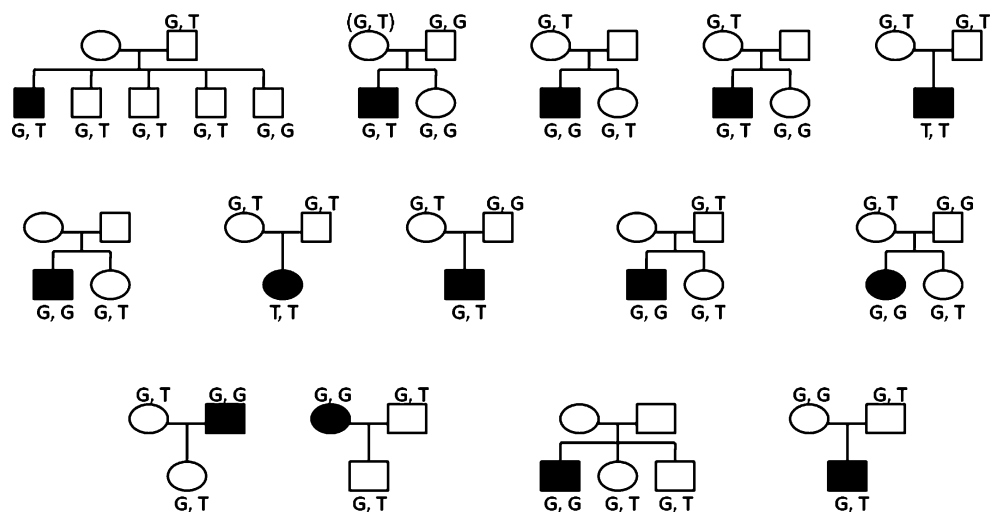
Peripheral leucocytes, DNA, and plasma were isolated from blood of subjects from the CVCR, at the time of recruitment, as previously described [10], in accordance with the principles of the Declaration of Helsinki with approval from the Institutional Review Boards of the University of Costa Rica and the University of Texas Health Science Center at San Antonio.

Plasma isolation and generation of lymphoblastoid cell lines

For plasma isolation, blood was spun within 30 min of collection at $3,000\times g$ for 3 min. Plasma was aspirated by pipette and frozen in 0.5-mL aliquots at -80°C until tested. Plasma was obtained from 45 individuals, from 14 separate families (Fig. 1), for measurement of autoantibodies and cytokine levels, to be used for quantitative trait analyses, as described below.

Lymphoblastoid cell lines (LCLs) were generated from leucocytes using LeucoPREP brand cell separation tubes (Becton Dickinson Labware) and transformed using Epstein–Barr virus (EBV). For all experiments, cells were grown in RPMI 1640 medium with 2 mM L-glutamine and

Fig. 1 Pedigrees of informative families for the *NRG1* $G>T$ polymorphism. Subjects in black were diagnosed as having a psychotic disorder ($N=14$), and subjects in white were diagnosed as unaffected ($N=31$). Subjects not containing allele information were not genotyped. Genotypes in parentheses were inferred. DSM-IV consensus diagnoses of the 14 affected individuals were as follows: schizophrenia, nine; schizoaffective depressed, one; bipolar disorder with psychosis, one; psychosis not otherwise specified, two; major depressive episode with psychosis, one



15% bovine growth serum at 37°C in a humidified 5% CO₂ chamber to a density of approximately 2×10^6 cells/ml.

Cell lines of unaffected unrelated individuals were selected for the experiments described below. All individuals had one first degree relative with psychosis. The cell lines used were from wild type (Val/Val, $N=6$; three males, three females, average age at blood draw 40 ± 20.8 year) and heterozygous *T* allele carriers (Val/Leu, $N=5$; two males, three females, average age at blood draw 44 ± 17 year).

All experiments described below were repeated at least twice. While conducting the experiments, the investigators were blinded to the experimental groups.

Autoimmune biomarker analysis

A commercial multiplex assay that concurrently estimates the levels of antibodies to 43 autoimmune biomarkers (Table 1) was used (Rules-Based Medicine Inc., Austin, TX, USA). This multiplex microbead assay measures proteins in a similar manner to standard sandwich ELISA, with comparable sensitivity and range [13]. Fifty microliters of plasma was used for analysis.

Fluorescence-activated cell sorting

For cytokine quantitation, a validated cytometric bead array system (BD Biosciences) [14] was used to simultaneously measure levels of six different human cytokines from plasma samples and LCL supernatants using fluorescently conjugated detection antibodies. The minimum limits of detection (pg/ml) are 3.6, 7.2, 2.5, 3.3, 3.7, and 1.9 for IL-8, IL-1b, IL-6, IL-10, TNF- α , and IL-12p70, respectively. Data were analyzed on a FACSCalibur flow cytometer using the BD CBA software. For IL-6, fluorescence-activated cell sorting (FACS) data were validated using the Human IL-6 ELISA Kit II (BD Biosciences).

RNA isolation and gene expression studies

RNA isolation from LCLs was carried out using the Trizol method (Invitrogen), and samples were cleaned up using the Qiagen RNeasy plus Kit. Quantitative PCR was carried out using the RT² Profiler PCR Array system (SABiosciences), using a 7900HT Standard Real Time PCR system (Applied Biosystems). For the pathway-based approach, we used the “Common Cytokine” array (PAHS-021, SABiosciences). Each array tests 83 different genes of interest (GOI) and five endogenous housekeeping genes (HKG) in a 96-well format. Significant array data were validated using PCR assays (SABiosciences) specific for individual cytokine genes (*IL-6*, *IL-4*), with beta-2-microglobulin (*B2M*) used as the endogenous HKG. Expression data were analyzed using the $\Delta\Delta C_t$ method.

Statistical analysis

The autoantibody and cytokine plasma levels were used as quantitative traits to test for association with the *NRG1* mutation in 14 informative CVCR families using FBAT. The program computes bi-allelic tests of association with quantitative traits, using the following commands: trait (Biomarker); offset 0.000; model additive; test bi-allelic; minsize 10; min_freq 0.000; P 1.000. Quantitative FBAT builds on the traditional transmission disequilibrium test, which compares the transmission of marker alleles from parents with affected offspring. With quantitative traits (i.e., levels of cytokines in plasma), the allelic transmissions among offspring who have high quantitative trait values are compared with transmission among offspring with low trait values [15, 16]. In other words, the quantitative trait takes the place of affected status as the phenotype of interest.

For in vitro analyses, two-tailed, unpaired Student *t* tests were used as appropriate. All results are expressed as mean \pm SD.

Results

Autoantibody and cytokine measurements in plasma from Costa Rican families

We first measured levels of 41 different autoantibodies (Table 1) in plasma from 14 CVCR families, informative for the *V>L* mutation. These families were recruited and genotyped for the *NRG1* mutation as previously described [10]. Of the 14 families, seven were part of the original study [10], and seven were new additional families, recruited at a later date. These 14 families were chosen, out of 206 available CVCR families, because they carried the *NRG1* mutation and the transmission of the rare *T* allele from parent to offspring could be inferred, and we had plasma samples available for all genotyped family members.

The families were comprised of 45 individuals; 14 homozygous normal, 29 heterozygous, and two homozygous rare (Fig. 1). Of the 45 individuals, 14 were affected and 31 were unaffected (Fig. 1). The DSM-IV diagnoses for affected individuals in these families were as follows: schizophrenia (nine), bipolar disorder type I with psychosis (one), schizoaffective disorder depressed type (one), major depressive episode with psychosis (one), and psychotic disorder not otherwise specified (two). The *T* allele was not found to be significantly associated with schizophrenia or psychosis in these 14 families by FBAT.

Levels of antibodies were measured in plasma from the 45 individuals by using a validated multiplex immunoassay developed by Rules-Based Medicine

Table 1 Quantitative FBAT analysis of autoantibody and cytokine levels in plasma of 14 families informative for the *NRG1* Val (G)>Leu (T) mutation

	Allele	<i>S</i>	<i>E(S)</i>	Var(<i>S</i>)	<i>Z</i>	<i>P</i>	
Antigen							
	Anti-nuclear	T	47.600	36.483	21.993	2.370	0.0177
	Beta-2 glycoprotein	T	358.90	298.81	4829.1	0.865	0.3872
	Complement factor 1q	T	165.70	139.53	186.13	1.918	0.0551
	Centromere protein B	T	44.800	34.907	22.310	2.095	0.0362
	Collagen type 1	T	2578.6	1847.0	188162.16	1.686	0.0917
	Collagen type 2	T	86.500	68.613	61.727	2.277	0.0228
	Collagen type 4	T	31.700	24.840	8.4890	2.354	0.0185
	Collagen type 6	T	171.60	130.49	309.76	2.324	0.0201
	Cytochrome P450	T	119.80	94.603	131.81	2.195	0.0281
	Double-stranded DNA	T	111.00	75.663	219.56	2.385	0.0170
	HSC	T	45.100	36.030	19.978	2.029	0.0424
	HSP	T	57.500	44.617	26.077	2.523	0.0116
	HSP	T	299.60	257.26	2028.2	0.940	0.3472
	HSP	T	55.900	42.800	37.917	2.127	0.0333
	HSP	T	275.00	224.32	824.64	1.765	0.0776
	HSP-90 beta	T	218.50	155.02	1652.7	1.561	0.1184
	Histone	T	70.400	56.323	47.464	2.043	0.0410
	Histone H1	T	66.000	51.650	40.717	2.249	0.0245
	Histone H2A	T	108.40	92.840	151.22	1.265	0.2057
	Histone H2B	T	70.900	84.043	975.67	-0.421	0.6739
	Histone H3	T	89.200	71.777	90.549	1.831	0.0671
	Histone H4	T	48.400	39.920	16.080	2.364	0.0180
	Insulin	T	55.400	45.610	25.070	1.955	0.0505
	JO-1	T	33.000	26.183	7.8220	2.437	0.0147
	Mitochondrial	T	57.200	43.883	33.633	2.296	0.0216
	Myeloperoxidase	T	57.900	46.250	24.365	2.360	0.0182
	PCNA	T	75.100	52.877	87.825	2.371	0.0177
	PM-1	T	39.000	29.540	13.090	2.615	0.0089
	Proteinase 3	T	89.900	69.307	66.156	2.532	0.0113
	RNP	T	52.200	40.497	20.960	2.556	0.0105
	RNP-A	T	224.70	170.57	875.63	1.829	0.0673
	RNP-C	T	89.748	75.863	51.894	1.927	0.0539
	Ribosomal P	T	42.600	32.947	14.726	2.516	0.0118
	Scleroderma 70	T	135.00	197.73	5313.8	-0.861	0.3894
	Smith	T	49.500	37.960	19.866	2.589	0.0096
	SSA	T	44.900	31.280	67.124	1.662	0.0964
	SSB	T	48.300	37.610	17.501	2.555	0.0106
	Thyroglobulin	T	88.800	56.960	439.72	1.518	0.1289
	Thyroid microsomal	T	155.90	114.21	330.98	2.291	0.0219
	T3	T	38.900	30.270	12.438	2.447	0.0144
	T4	T	15.270	12.338	2.041	2.052	0.4017
Cytokine							
	IL12p70	T	29.189	23.935	4.770	2.406	0.0161
	TNF	T	29.839	24.498	5.472	2.283	0.0224
	IL10	T	29.009	23.196	6.267	2.322	0.0202
	IL6	T	36.499	30.615	7.811	2.105	0.0352
	IL1b	T	40.307	33.691	9.824	2.111	0.0347
	IL8	T	74.444	63.779	42.162	1.642	0.1005

P values for markers showing higher than expected levels of antigen associated with the *NRG1* T allele in 14 informative families are shown in bold.

Cytokine levels were measured in plasma using a validated cytometric bead array system (BD Biosciences). Autoantibody levels were measured in plasma using a validated multiplex immunoassay developed by Rules-Based Medicine (www.rulesbasedmedicine.com)

S test statistic, *E(S)* expected value of the test statistic under a null hypothesis of no association

(www.rulesbasedmedicine.com). The mean values of antibody levels for wild type and mutants are listed in supplemental Table 1. For quantitative analysis, antibody levels were normalized using natural log. We used the normalized levels as quantitative traits to perform a quantitative family based association test (qFBAT) to evaluate association of the *NRG1* mutation with antibody levels. Quantitative FBAT utilizes the levels of biomarkers as the phenotype of interest, and therefore, analysis is done without regard to affected status. This is of importance because the goal of this study is to determine the association of the *NRG1* mutation with immune dysregulation, independent of psychiatric diagnosis. Higher than expected levels of antibodies to 25 different autoimmune markers were significantly associated with the *NRG1* mutation (*T* allele; Z value >2 , $P<0.05$) in the 14 families (Table 1). Although the individual P values are not corrected for multiple testing, the fact that greater than half of the markers, 25 out of 41, showed positive association with the mutation suggested a “signature” of abnormal immune regulation in these families, leading to a generalized immune activation as manifested by increased development of antibodies against endogenous antigens. There is a possibility that the observed increased antibody levels could be due to high schizophrenia loading in these families. However, an inspection of the family pedigrees reveals that there is an equal loading of psychiatric diagnosis on *G G* and *G T* individuals in these families (Fig. 1).

Next, we surmised that if indeed there was a generalized immune dysregulation in carriers of the *V>L* mutation, levels of circulating immune mediators (cytokines) should also be elevated in the 14 families. We measured levels of six different cytokines (IL-1b, IL-6, IL-10, IL-8, IL-12p70, and TNF- α) in plasma from the 45 individuals, using a cytometric bead array system followed by FACS analysis. The mean cytokine levels for each genotype group are listed in supplemental Table 1. Statistical analysis using qFBAT indicated that higher than expected levels of IL-1b, IL-6, IL-10, IL-12p70, and TNF- α were significantly associated (Z value >2 , $P<0.05$) with the *NRG1* mutation in these 14 families (Table 1).

Immunological profiling of lymphoblastoid cell lines

Considering that multiple confounding factors could affect measurements of antibody titers and cytokines *in vivo*, we sought to obtain more direct evidence for an effect of *NRG1* *in vitro* by comparing the immunological features of EBV-transformed B cells derived from heterozygous vs. wild-type individuals. The study of LCLs is uniquely suited to the investigation of genetic polymorphisms in immune activation. *In vitro*, the EB virus infects resting (naive or

memory) B lymphocytes and transforms them into immortalized, polyclonal LCLs. Regardless of their origin, most EB virus genome-carrying cell lines are thought to contain B cells that are “frozen” at the lymphoblast stage of differentiation [17]. In other words, the analysis of the immune phenotype of LCLs can provide information about the overall immune activation of the donor at the time of sample collection. Furthermore, LCLs are ideal for the study of the effects of polymorphisms in the transcriptome. Concurrent genome-wide SNP and expression studies revealed that genes expressed in LCLs are enriched in gene ontology categories of immune response, and there is a significant heritability for these traits, emphasizing the value of the use of LCLs for the study of genetic variation in immunity [18]. In addition, data mining mRNA expression arrays of LCLs from 15 CEPH/Utah families revealed expression of *NRG1* [18], suggesting that LCLs could be informative about the effects of the *NRG1 V>L* mutation in immune dysregulation. [Gene Expression Omnibus database, DataSet GDS1048, (<http://www.ncbi.nlm.nih.gov/geo>)].

Measurement of cytokine expression levels in LCLs

We first measured expression of IL-8, IL-1b, IL-6, IL-10, TNF- α , and IL-12p70 in supernatants from LCLs of wild-type (Val/Val, $N=6$) and heterozygous *T* allele carriers (Val/Leu, $N=5$). The cell lines were chosen from unrelated, unaffected individuals, each with a first degree relative with psychosis. Because the family loading for psychosis is equal for all samples and the individuals themselves do not suffer from any psychiatric disorder, we can directly investigate the effect of the *V>L* mutation on cytokine expression, without the confounding factor of an existing psychiatric disorder. The five *T* allele carriers were chosen from the 14 informative families described above, each from a different family. As described, only two homozygous mutation carriers have been identified in the CVCR population. Both of these individuals have a schizophrenia diagnosis. Given that the present experimental design excluded affected individuals, for all experiments described below, only heterozygous carriers were used.

Equal numbers of cells were plated overnight, and cytokine levels were evaluated from supernatants, 24 h after plating, using FACS. Cell counts were not found to be different between wild type and mutants 24 h after plating (data not shown). We found that, compared with LCLs from wild-type carriers, LCLs from *T* allele carriers produced significantly higher levels of proinflammatory cytokines (Fig. 2a). TNF- α , IL-6, and IL-8 showed significantly higher levels in the mutants compared with controls (two-tailed Student *t*, $P=0.0187$, 0.0166 , 0.0323 , respectively).

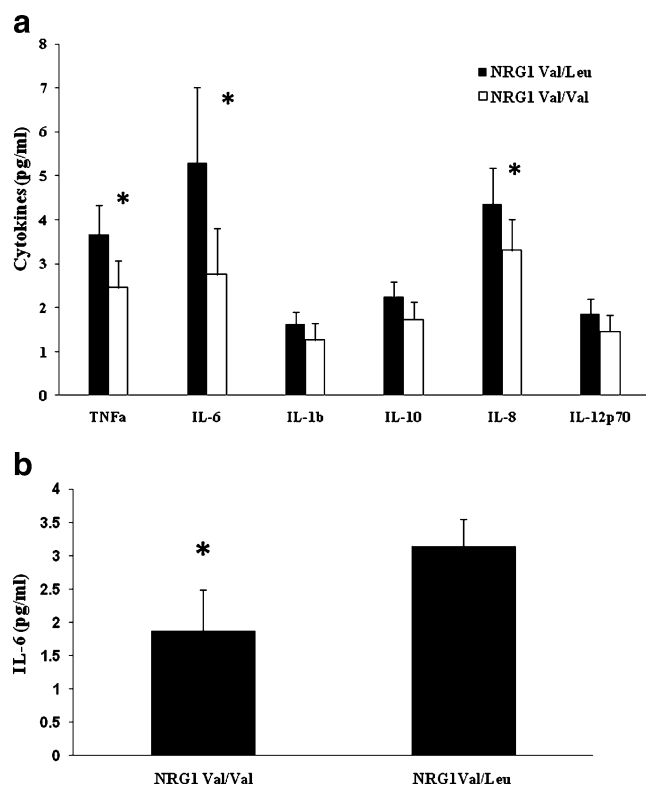


Fig. 2 Expression of cytokines in lymphoblastoid cell lines. **a** Measurement of cytokine levels using FACS in cell supernatants from LCLs of carriers of the *NRG1* mutation compared with controls. $P=0.0187$, 0.0166 , and 0.0323 , for TNF- α , IL-6, and IL-8, respectively (two-tailed Student *t*). **b** Measurement of IL-6 levels using ELISA in cell supernatants from LCLs of carriers of the *NRG1* mutation and controls. $P=0.008$ (two-tailed Student *t*). Val/Leu, $N=5$ and Val/Val, $N=6$. All concentration values are expressed in pg/ml. Data represent log-transformed values. Asterisks represent significant P value

We validated the IL-6 results using an IL-6-specific ELISA. IL-6 supernatant levels, normalized to total protein content, were observed to be significantly higher in mutants compared with controls, confirming the results obtained in the six-cytokine kit (two-tailed Student *t*, $P=0.008$) (Fig. 2b).

Next, a cytokine gene array (SABiosciences) was used to evaluate 83 cytokine mRNA levels by quantitative PCR in the same cell lines as above, wild-type ($N=6$) and *T* allele

carriers ($N=5$). Four genes, *IL-4*, *IL-6*, *IFNA2*, and *BMP8B*, showed a significant difference between the mutants and controls (Table 2).

The *IL-4* and *IL-6* results were validated using specific single gene assays. cDNA generated from each individual cell line was assayed in duplicate using *B2M* as the endogenous housekeeping control. We observed a 4.39-fold down-regulation of *IL-4* and a 35.46-fold up-regulation of *IL-6* in mutants compared with controls (two-tailed Student *t*, $P=0.014$ and 0.0011 for *IL4* and *IL-6*, respectively) (Table 3).

Discussion

Because of its multiple isoforms and various functional roles, NRG1 has been implicated in the pathophysiology of a wide variety of disorders, including breast cancer [19] schizophrenia and bipolar disorder [20] in human studies, and atherosclerosis [21], myocardial dysfunction [22] and multiple sclerosis [23] in animal models. Interestingly, immune system dysregulation has been implicated as a risk factor in all of these disorders. Inflammation has been associated with poor prognosis in breast cancer [24]. Proinflammatory imbalance and uncontrolled immune response have been postulated as the cause of acute myocardial infarction [25]. In schizophrenia, immune system abnormalities such as increased expression of proinflammatory cytokines and high levels of autoantibodies have been repeatedly reported [7, 8]. Multiple sclerosis is characterized by immune system-mediated demyelination and axonal loss. In an animal model of multiple sclerosis, Marchionni et al. demonstrated that treatment with recombinant NRG1 caused greater remyelination and decreased relapse rate, compared with untreated controls [23]. Increased expressions of myelin basic protein and IL-10 were also observed. The modulation of myelination by NRG1 in the CNS (but not in the PNS) is thought to be independent of NRG1 ErbB signaling [26].

Table 2 Comparative mRNA profiles in NRG1 Val/Val and Val/Leu cell lines

Gene	Average Δ Ct mutants	Average Δ Ct controls	$2^{-\Delta$ Ct mutants	$2^{-\Delta$ Ct controls	Fold difference mutants/controls	<i>t</i> test P value
<i>IL-4</i>	16.43	14.79	1.1E-05	3.5E-05	↓ 3.10	0.0340
<i>IL-6</i>	8.61	13.05	2.6E	9.6E	↑ 27.08	0.0404
<i>IFNA2</i>	11.91	9.60	2.6E	1.3E	↓ 5.00	0.0505
<i>BMP8B</i>	11.29	12.94	4.0E	1.3E	↑ 3.14	0.0459

Data were generated using a common cytokine gene array (PAHS 021, SABiosciences). Fold up or down expression in mutants compared with controls is denoted by arrows. Genes shown here are those which showed significant evidence of differential mRNA expression in mutants (Val/Leu, $N=5$) compared with controls (Val/Val, $N=6$): interleukin-4 (*IL-4*), interleukin-6 (*IL-6*), interferon-A2 (*IFNA2*), bone morphogenetic protein 8b (*BMP8*). Avg Δ Ct=(Ct(GOI)-Ave Ct (HKGene)). Ct=cycle threshold, minimum number of PCR cycles needed to detect expression. High Ct=low expression; low Ct=high expression

Table 3 mRNA expression levels of IL6 and IL4 in NRG1 Val/Val and Val/Leu cell lines

Gene	Average ΔCt controls	Average ΔCt mutants	$2^{-\Delta\text{Ct}}$ controls	$2^{-\Delta\text{Ct}}$ mutants	Fold difference mutants/controls	<i>t</i> test <i>P</i> value
<i>IL-4</i>	15.90240	18.04351	1.62E-05	3.70E-06	↓ 4.39	0.0150
<i>IL-6</i>	13.82899	8.681128	6.87E-05	2.43E-03	↑ 35.46	0.0011

Fold up or down expression in mutants (Val/Leu, $N=5$) compared with controls (Val/Val, $N=6$) is denoted by *arrows*. Avg $\Delta\text{Ct}=(\text{Ct}(\text{GOI})-\text{Ave Ct}(\text{HKG}))$. Ct=minimum number of PCR cycles needed to detect expression. High Ct=low expression; low Ct=high expression

Our present results suggest the possibility that the NRG1 $V>L$ mutation may be involved in causing immune system dysregulation, which then, in combination with different specific sets of genes and environmental factors, may lead to development of a particular disorder. The NRG1 $V>L$ mutation was first identified in a genetic study searching for schizophrenia-causative mutations [10]. Although the NRG1 gene is one of the most promising schizophrenia candidate genes identified to date, no definite causative mutations within this gene have been identified. Therefore, research aimed at determining the functional role of NRG1 polymorphisms is an area of much interest. A deeper understanding of the biological functionality of NRG1 variants could help to understand the role that NRG1 may play in different pathological conditions.

In this study, we aimed to determine whether the $V>L$ polymorphism could be associated with immune system alterations. Given that this mutation occurs in the transmembrane region of NRG1 and knocking out this region has been shown to cause schizophrenia-like behavior in mice [27], we focused exclusively on this transmembrane mutation. However, it is highly possible that other NRG1 variants, as well as SNPs within other schizophrenia candidate genes (i.e., DISC1, dysbindin, and COMT), may also be associated with immune system dysregulation. To our knowledge, SNPs in schizophrenia candidate genes other than cytokines have not been previously reported to be associated with alterations in the immune system. We hope our present results will encourage other researchers to investigate this possibility.

We observed statistical evidence of increased plasma autoantibodies and proinflammatory cytokines, including IL-6, TNF- α , and IL-1b in carriers of the $V>L$ mutation. Polymorphisms within *IL-6* and *IL-1b* have been associated with schizophrenia [28, 29]. Interestingly, it has been shown that polymorphisms in *IL-1b* and NRG1 genes interact statistically with each other and are linked to schizophrenia [29]. Increased levels of proinflammatory cytokines, in particular IL-6 and TNF- α , have been repeatedly observed in schizophrenia patients [8]. IL-6 has also been implicated in worsening of autoimmune disorders by causing blood–brain barrier disturbances and increased IgG secretion [8]. In a large linkage study of Danish National Registers, a 45% greater risk of developing

schizophrenia in families with a history of autoimmune disorders was reported [9]. Furthermore, high levels of autoantibodies, including anticardiolipin, anti-histone, anti-DNA, and anti-nuclear antibodies, have been reported in schizophrenic patients. Serum antibodies against brain-specific regions such as the hippocampus, amygdala, and frontal cortex have also been reported [8]. Na and Kim [30] reported reduced levels of IL-4, an anti-inflammatory cytokine, in serum of schizophrenia patients compared with controls. This correlates with our present data showing reduced *IL-4* expression in cell lines of carriers of the $V>L$ mutation, compared with wild-type carriers (Fig. 2). Altogether, these studies support the hypothesis that an overactive immune system could be an underlying causative factor in schizophrenia development. Our current findings suggest that this over-activity could be in part due to the NRG1 $V>L$ mutation. Further studies with additional and larger sets of samples from different populations need to be performed in order to corroborate these results.

Investigation of the specific mechanism by which the $V>L$ mutation leads to increased immune activation is currently underway in our laboratory. One possibility is that the change of $V>L$ alters the proteolysis of NRG1 transmembrane proteins, resulting in different availability of bioactive extracellular and intracellular domains of NRG1, which could possibly lead to immune dysregulation. The NRG1 transmembrane domain interacts with metalloproteinases, such as β and γ secretase, which form the bioactive extracellular and intracellular domains of the proteins. The cleaved extracellular NRG1 domain interacts with ErbB receptors and activates numerous downstream pathways, such as the AKT and ERK pathways, which are involved in regulation of gene expression [31], and in leukocyte activation and survival. In several different strains of mice that exhibit lupus-like phenotypes, the level of phosphorylation of AKT and ERK in unstimulated B cells was significantly elevated [32]. Moreover, the ERK pathway has been shown to regulate cytokine expression [33].

The intracellular NRG1 domain, formed via γ secretase-mediated cleavage, migrates to the nucleus and acts as a transcription factor, regulating mRNA expression of genes such as *BCL-X_L*, *BAK*, *RIP*, and *OCT-3* [12]. Importantly, a

recent study showed that the *NRG1 V>L* mutation reduces γ -secretase cleavage in COS-1 cells [11], suggesting that this mutation alters the function of the *NRG1* protein. Differential availability of intracellular *NRG1* associated with the *V>L* mutation may result in dysregulation of the mechanisms underlying elimination of self-reactive B cells, which are mostly dependent on the counterbalancing effects of pro-apoptotic proteins such as BAK and anti-apoptotic proteins such as BCL-XL [34]. Persistence of self-reactive B cells may lead to autoantibody production and in certain cases full-blown autoimmune disorders [34].

Given that both the extracellular and intracellular *NRG1* pathways are involved in regulation of gene expression, it is possible that cytokine expression is regulated by either, or both, of these pathways. Interestingly, IL-6 and IFN- γ have recently been shown to induce *NRG1* cleavage in endothelial cells and enhance angiogenesis via *NRG1* signaling in vitro [35]. Therefore, it is possible that the increase in IL-6 expression we have observed in the present study may be a feedback mechanism compensating for reduced *NRG1* cleavage due to the *V>L* mutation. Further experiments need to be carried out to determine the exact mechanism by which *NRG1* may regulate cytokine expression.

Given the small sample sizes used in this study, our present results must be interpreted with caution and should be replicated by independent samples. In addition, studies investigating the potential association of other *NRG1* polymorphisms with immune system dysregulation are warranted.

In conclusion, our study is the first to report statistical association between a mutation in *NRG1* and immune system dysregulation. These findings could aid in the understanding of the molecular mechanisms of inflammatory regulated disorders in which *NRG1* plays an important role.

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Conflict of Interest The authors declare no conflict of interest.

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